Immobilization of biocatalysts on solid supports for analytical applications

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IMMOBILIZATION OF BIOCATALYSTS ON SOLID SUPPORTS FOR ANALYTICAL APPLICATIONS

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ABSTRACT

Enzyme sensors for diagnostic and environmental applications have been developed by immobilizing biocatalysts onto the active solid surface of physico-chemical sensors. The performances of a biosensor are mainly related to the properties of the bioreceptor material and the techniques used for its immobilization onto the transducer. Different enzyme immobilization techniques will be presented: immersion, direct binding, reinforced membranes and finally aerosol vaporisation that combines adsorption and covalent binding of proteins to obtain ultrathin bioactive films.

INTRODUCTION

A biosensor can be considered as a combination of a bioreceptor, the biological component, and a transducer, the detection system. The total effect of a biosensor is to produce an electrical signal from a biological event. The bioreceptor ensures molecular recognition, and may transform the analyte in some way. This localized modification is generally made via an immobilized enzyme, which catalyses the conversion of substrate into a product that is detectable by the transducer. This is the case for enzyme biosensors. Therefore, an enzyme biosensor constructed from association of a transducer and a thin enzymatic layer, is normally used to measure the concentration of a substrate. An extension of this definition is that the concentration of any substance can be measured provided that its presence affects the rate of an enzymatic reaction; this is especially true for enzyme inhibitors.

MATERIALS

1. Immersion method

1. Glass electrode (micro-electrode can also be used).
2. Sodium phosphate buffer solution 0.01M, pH 7.
3. Enzyme solution: 1mg urease EC.3.5.1.5 from *Jack beans* (≈ 800 units per mg protein) in 1mL sodium phosphate buffer solution.
4. Bovine Serum Albumin (BSA) 30% in water.
5. Glutaraldehyde 25% aqueous solution as cross-linking agent.

2. Direct binding method

1. Glass electrode with flat tip of 2.5 cm^2 in area.
2. Sodium phosphate buffer solution 0.01M, pH 7.
3. Enzyme mixture: 0.2 mg butyrylcholinesterase EC.3.1.1.8 from *Horse serum* (≈ 500 units per mg protein) in 0.1mL phosphate buffer solution mixed with 0.1 mL bovine serum albumin (30% aqueous solution).
4. Glutaraldehyde solution (2.5% in water) as cross-linking agent.

3. Reinforced membranes

1. Glass electrode equipped with a clip device.
2. A nylon net (5 cm x 4 cm) of 140 µm meshes.

3. Two teflon sheets (5 cm x 4 cm), 5 cm in thickness.
4. Sodium phosphate buffer solution 0.01M, pH 7.
5. Enzyme mixture: 2 mg butyrylcholinesterase EC.3.1.1.8 from *Horse serum* (≈ 500 units per mg protein) in 1 mL phosphate buffer solution mixed with 1 mL bovine serum albumin (30% aqueous solution).
6. Glutaraldehyde solution: 2.5% in water.

4. Aerosol vaporisation

1. Glass electrode (type XG from Tacussel).
2. Sodium phosphate buffer solution 0.01M, pH 7.
3. Enzyme solution: 4 mg penicillinase EC.3.5.2.6 from *Bacillus cereus* (≈ 400 units per mg protein) in 1mL of phosphate buffer solution 0.01M, pH 7.
4. Glutaraldehyde solution: 2.5% in water.
5. Air-brush (type Atrium from Lefranc Bourgeois) using nitrogen under a pressure of 1.5 bar.

METHODS

1. Immersion method

1. Rinse the pH glass electrode tip with acetone and with distilled water then dry it with filter paper.

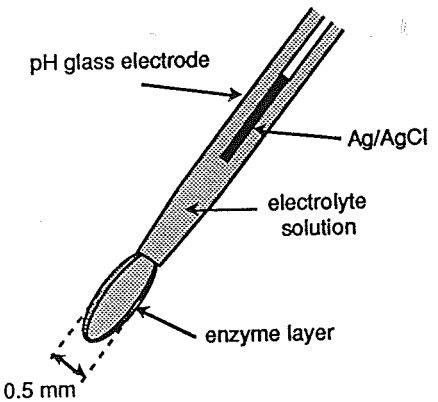


Figure 1. Immersion method used to immobilize enzyme on a micro-pH electrode.

2. Dip the whole bulb in a mixture of 1mL enzyme solution, 1mL albumin solution and 0.07 mL glutaraldehyde 25% solution for 5 seconds, so that the bulb of the electrode is completely covered.
3. Rotate the electrode for 15 minutes to give a homogeneous enzymatic layer which coagulates after this period of time (Fig. 1).
4. Rinse the electrode with a phosphate buffer solution to elute any excess cross-linking agent.

2. Direct binding method

1. Rinse the flat tip of the pH glass electrode with acetone and with distilled water then dry it with filter paper.
2. Turn the electrode upside down so that the flat part of the electrode is horizontal (Fig. 2).
3. Spread 10 µL of the enzyme mixture on the flat part of the electrode, then 10 µL of glutaraldehyde solution.

4. Perform crosslinking during 15 minutes at ambient temperature.
5. Rinse the electrode with a phosphate buffer solution to elute any excess cross-linking agent.

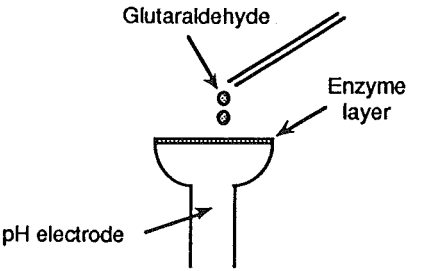


Figure 2. Immobilization of enzymes on a pH electrode by the direct binding method.

3. Reinforced membranes

1. Lay the nylon net on one of the teflon sheets.
2. Mix 0.2 mL of butyrylcholinesterase solution with 0.2 mL of glutaraldehyde solution.
3. Rapidly spread this mixture on the whole surface of the nylon net.
4. Cover this surface with the other teflon sheet and press firmly.
5. Remove the enzyme nylon net after 30 minutes crosslinking.
6. Rinse the enzyme nylon net with a phosphate buffer solution to elute the cross-linking agent.
7. Cut the net in small circles with appropriate membrane dimensions.
8. Clip one membrane on the tip of the pH electrode (Fig. 3).

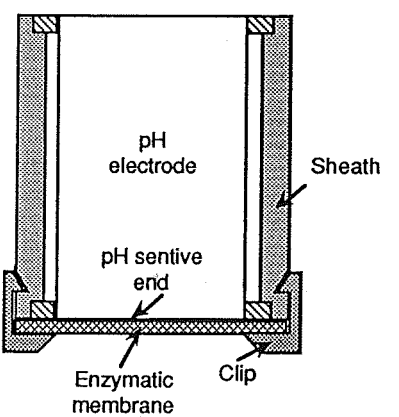


Figure 3. Biosensor with a reinforced enzymatic membrane clipped onto a transducer.

4. Aerosol vaporisation

1. Rinse the tip of the pH glass electrode with acetone and with distilled water.
2. Dip it alternatively three to four times in 0.1 M HCl and 0.1 M NaOH for 0.5 h each time.
3. Wash it with water and then dry it with filter paper.
4. Immerse it in an enzyme solution for about 20 minutes to ensure the adsorption of the enzyme on the glass.
5. Dry the pH electrode at 4 °C during 20 minutes and set it to rotate horizontally.

6. Vaporize the glutaraldehyde solution during 3 seconds onto the sensitive end of the pH electrode from a distance large enough to prevent formation of droplets (Fig. 4).
7. After cross-linking, rinse the enzyme electrode with a phosphate buffer solution.

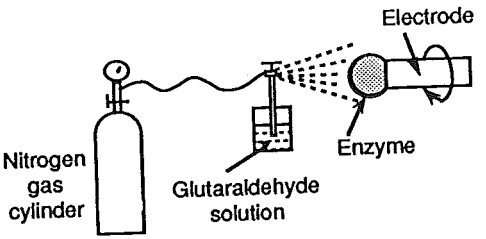


Figure 4. Deposition of thin enzymatic membranes in the construction of glass enzyme electrodes.

RESULTS AND DISCUSSION

Many electrochemical transducers have been used to monitor the enzymatic reaction by potentiometric or amperometric measurements of the product or co-substrate concentration. When the enzymatic reaction results in a change in pH, a pH electrode can be used as a potentiometric transducer.

A schematic representation of an enzyme biosensor based on pH electrode is given in Figure 5. The sensitive surface of the pH electrode is in contact with an enzymatic layer, and is immersed in a solution containing the substrate. The substrate migrates towards the interior of the layer and is converted into reaction products, with proton H^+ production or consumption, when it reacts with the immobilized enzyme.

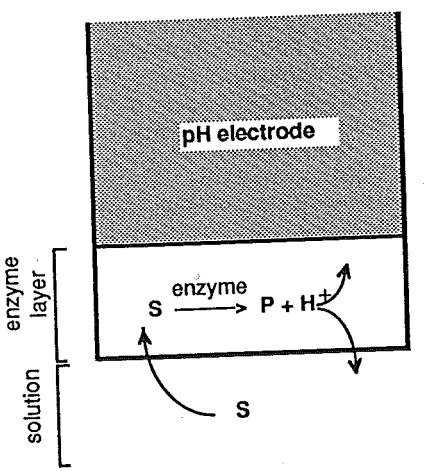


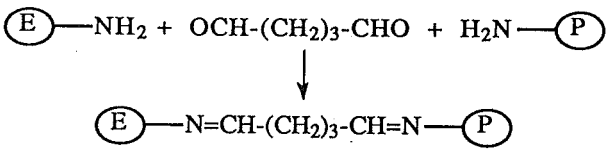
Figure 5. Schematic representation of the diffusion of the substrate S and the products P and H^+ in the enzymatic layer on a pH electrode.

The performances of enzyme electrodes will largely depend upon the immobilisation of the enzyme onto the electrode to ensure maximal contact and response. Enzymes can be immobilized by physical entrapment because they are proteins with high molecular masses and sizes large enough to be trapped in polyacrylamide gels. It is also possible to spread a solution of enzyme over the surface of an electrode and cover the whole assembly with a

dialysis membrane [1] to prevent protein diffusion. Physical entrapment of enzymes is now rarely used because of the leaching of enzyme and the loss of enzymatic activity. Chemical immobilization techniques are preferred because they involve covalent bonds, which ensure a greater long-term enzymatic stability.

Cross-linking is a chemical immobilization method [2] that uses a bi- or multifunctional agent to form a bridge between different biocatalytic species or proteins. The process results in a considerable increase in molecular mass, and the compounds or aggregates formed are insoluble. It is possible to crosslink molecules of the same enzyme, or to coreticulate two or more different proteins (enzyme with enzyme, enzyme with protein, or more than one enzyme with a load protein such as bovine serum albumin). Coreticulation is a very important process because it facilitates multienzymatic immobilization. The use of a load protein like albumin improves enzymatic activity and mechanical properties of the membranes produced because of the better mass distribution of the various proteins.

Glutaraldehyde is the usual choice for a crosslinking agent. This bifunctional agent has two aldehyde groups at its extremities which react with the amine group on the enzyme or protein, forming derivatives that are analogues of Schiff bases.



Several techniques were used to construct enzyme electrodes. The enzyme can be immobilized onto the electrode by dipping the electrode in a solution containing the enzyme with a crosslinking agent (immersion method). The immersion method is simple, and suitable for immobilizing enzymes onto most transducers particularly small transducers for *in vivo* clinical analysis. However, it requires a large amount of enzymes. Whenever the enzyme is available in small amounts, then it is better to immobilize it directly [3] on the tip of the pH electrode (direct method). This method is also applied for expensive enzymes.

Biological materials have limited lifetimes and so removable active membranes are useful. Hence the idea of immobilizing an enzyme on a membrane, and attaching the membrane to the transducer. The step also avoids the constraints of enzymatic immobilization, and allows mass production, which improves the reproducibility of the biosensor signal. Reinforced membranes have a fabric base, which ensures the mechanical properties of enzymatic membranes [4] and facilitates their manipulation. The enzyme is immobilized on a large area of nylon net from which small disks of enzymatic membrane are cut in the exact dimensions of the transducer. These membranes come from the same batch and the same immobilization, and thus have reproducible activity. Membranes obtained in this way can be clipped onto the transducer [5], which is much better than using screws which may twist or tear the membrane.

Alternatively, prefunctionalized membranes (Immunodyne produced by Pall Industrie, or Ultra-Bind produced by Gelman Sciences) can be used instead of nylon nets. They are simply immersed in a solution containing the chosen enzyme to obtain an enzymatic membrane [6]. However leaching of enzymes has been observed with changes in pH or ionic strenght.

A large number of applications require biosensors with a rapid response. Examples include alarm systems, in which urgency is vital, and flow injection analysis (FIA), in which a rapid response can increase the sample throughput and reduce the cost of analysis. Biosensor response times are very dependent on the thickness of the active layer. Aerosol vaporization of dissolved compounds deposits films that are thin and homogeneous. This technique was applied to the direct binding of enzymes onto pH electrodes by vaporizing a cross-linking agent onto a protein that had been previously adsorbed onto the sensitive surface of a transducer [7].

CONCLUSION

1. Immersion method

- Immersion method can be used to immobilize enzymes or proteins onto electrodes of all shapes.
- The enzyme layer thickness obtained by this method depends on the viscosity of the enzyme solution.
- The correct binding time is to be adjusted: after too short a time, the coating is fragile and easily torn; if too long, it is less active and adherence is poor.
- The binding time depends on albumin and glutaraldehyde concentration.
- This method is enzyme consuming because the enzyme solution with glutaraldehyde cannot be reused since it coagulates at the same time as the enzymatic layer.

2. Direct binding method

- This technique can also be applied to a spherical tip pH electrode.
- In this case, take care to spread the enzyme and the glutaraldehyde solution regularly on the whole surface of the sensitive end.
- This method is rather manual since particular care is taken to have an even layer.

3. Reinforced membranes

- Other fabrics than nylon net could be used provided that the meshes are not too wide to have a continuous enzyme layer.
- Reinforced enzymatic membranes are recommended in stirred solutions.
- This technique is well adapted to produce ready-made enzymatic membranes.

4. Aerosol vaporisation

- Films obtained in this way are extremely thin (1-2 μm).
- The resulting biosensors have very short response times (5-10 seconds).
- This procedure can be used to immobilize enzymes on a variety of transducers.
- It is also suitable for mass production of enzyme biosensors.

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